Journal of Animal & Plant Sciences, 32(3): 2022, Page: 784-790
ISSN (print): 1018-7081; ISSN (online): 2309-8694
http://doi.org/10.36899/JAPS.2022.3.0480

IMAGING OF PHYTOSPHERE COLONIZATION BY GFP (GREEN FLUORESCENT PROTEIN) EXPRESSED EPIPHYTIC PHYLLOSPHERIC BACTERIA

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ABSTRACT

Bacteria living on the aerial parts of plant come from rhizosphere or/and above ground environment. Leaf associated microbes flourish in harsh conditions, adaptable to other environments, have ability to move across plant and colonize the other parts of plants as well. Imaging of GFP-labelled epiphytic phyllospheric bacteria *Pseudomonas* sp. MehA-P42 on root, stem and leaves of two sunflower hybrids was done by fluorescence microscopy and CLSM (confocal laser scanning microscope). Phyllo-epiphytic bacteria applied to seeds not even reached and colonized the destined aerial parts but also colonized roots and stem surface of plants. The internal localization (in the inter- and intra-cellular compartments of leaves, stems and roots) of GFP-labeled *Pseudomonas* sp. MehA-P42 was revealed by CLSM. Phyllo-epiphytic bacteria isolated from one sunflower variety (Hysun-39) established an association with another variety (Hysun-33) through colonization in and on the root stem and leaves surface. Study indicated the movement of plant associated bacteria along phytosphere as well as the ability of phyllospheric bacterial isolates to adapt the environment and flourish on the plants other than the native. This supports the field application of the phyllospheric bacterial isolates to seeds, roots of plant or in rhizosphere as plant stimulator and biocontrol agent.

Key Words: GFP, Phyllosphere, colonization, imaging, fluorescent microscopy, CLSM

Published first online October 20. 2021 Published final May 30. 2022

INTRODUCTION

Due to the inherently open existence of the leaf system, it is theorised that the source of microbial flora that grows on the leaf differs. With the assistance of insects, aerosols and soil, the bacterial transmission may be possible (Koskella, 2020). Plant roots are surrounded by soil, which is the most incredibly rich source of microbial diversity and the most likely origin of many of the microbes that harbour plants (Herrera Paredes and Lebeis, 2016). There is a strong difference in complexity and composition between the rhizospheric and phyllospheric microbial populations, even though certain microbial genera are shared between the rhizosphere and phyllosphere (Bringel and Couée, 2015). The microbial population of the rhizosphere and its composition vary greatly from the phyllosphere associated microbes. The microbial communities of aboveground plant components such as flowers, fruiting bodies and leaves, however, are pretty much like the microbial flora of the soil from which they sprout rather than being identical to each other. Soil is known as a microbe reservoir for both above and below ground plant components (Bao *et al.*, 2020). The current research discusses the perception of the position of phyllospheric bacteria in different parts of the phytosphere, i.e. the roots, stem and leaves, as well as the transfer of bacteria from the roots to the leaves. Another point to be considered is that seed or soil source is used by most plant-growth-promoting bacteria (PGPB) and rhizosphere competence is seen as a key factor in determining the efficacy of PGPB in promoting both plant growth and disease control. In addition, discrepancies due to changes in the capacity of beneficial microorganisms to colonise the rhizosphere under different circumstances have also impeded their field application (Compant *et al.*, 2005; Bringel and Couée, 2015). Due to the concern about their competence in the rhizosphere, the application of phyllospheric bacteria to seeds has been challenged. In this study, this hypothesis is been tested that phyllospheric bacteria transmitted from soil to above parts of plants. The bacterial strain isolated from one plant variety can colonize the other or not.

MATERIALS AND METHODS

Selection of bacteria and GFP labelling of bacteria: Phyllospheric bacteria *Pseudomonas* sp. MehA-P42 (accession no. MW054553) isolated from the leaf surface of sunflower hybrid variety Hysun-39. GFP protein was introduced in the bacteria with the aid of Bio-Rad pGLO Bacterial Transformation and GFP Kits. Gene expression was confirmed by observing arabinose containing bacterial plates under the UV.

Bacterization and plant growth: Bacteria were grown in N-Agar at 37℃ for 24 hours and OD_{600} was set at 1. Seeds of two sunflower hybrids Hysun-33 and Hysun-39 were separately soaked in the bacterial culture for 30 minutes, sown in sterile soil microcosm and allowed to grow for 21 days in a growth chamber (16h photoperiod with light intensity 50 µmol m^{-1} s^{-1} and 30°C temperature). Plants were removed from soil microcosm, soil particles were washed away, and sections of root, stem and leaves were cut for imaging.

Fluorescent microscopy and Confocal laser scanning microscopy (CLSM): Fluorescent microscopy of plant parts was done with Olympus fluorescent microscope. GFP was excited by the 488 nm excitation filter and emission peak detected at 510 nm. Confocal laser scanning microscopy of root, stem and leaf sections was conducted using an excitation laser (Argon laser) of 488 nm and gathering the emission band of 500-550 nm for GFP fluorescence with the Leica SP2 with 4 laser system (Leica) (Fan *et al.*, 2011).

RESULTS

In this study, epiphytic bacteria *Pseudomonas* sp. MehA-P42 isolated from the leaves of *Helianthus annuus* was introduced with GFP protein with the aid of a pGLO bacterial transformation kit. Gene expression was confirmed by observing arabinose containing bacterial plates under the UV. The cells expressing the GFP protein showed green fluorescence in UV light. However, the cells on kanamycin containing plates did not show fluorescence in the absence of arabinose in media (Figure 1). No fluorescence was observed in the absence of arabinose on plates containing kanamycin as a marker. The stability of the GFP protein was assured by re-streaking GFP expressing protein after 5 days interval in 5 rounds and the expression of GFP protein was observed as bacteria fluoresce under UV in the presence of arabinose and did not show fluorescence in the absence of arabinose in media.

To find out whether the bacteria moved from the rhizosphere to the phyllosphere or not, seeds of two varieties of *Helianthus annuus* Hysun-33 and Hysyn-39 were soaked for 30 minutes in suspension (10^6 CFU/ml) of GFP labelled *Pseudomonas* sp. MehA-P42 (phyllospheric epiphytic bacteria) for the appropriate bacterial adherence to seeds. Seeds were sown in the sterile soil microcosm and allowed to grow in controlled conditions (16h photoperiod and 30°C temperature). Bacterial colonization on the phytosphere (roots, stem and leaves) was examined under a fluorescence microscope and confocal laser scanning microscope (CLSM) after 21 days of growth. Imaging of different plant parts assured the localization of bacteria on plant surface from roots to leaves via the stem.

Imaging of different plant parts under a fluorescent microscope revealed the localization of cells (figure 2). Bacterial adherence and localization on the surface of roots, stem and leaves were observed. Bacteria were observed as green in colour, however, the plant part having chlorophyll (leaf portion) was observed red under the microscope. No fluorescent bacterial cells were observed on uninoculated plant surfaces of both plant varieties. On the surface of the stem of Hysun-33, bacteria were seemed to appear in a linear manner. By observing the green fluorescence patches on the leaf surface, the localization of bacteria on the surface was conceived. The intensity of fluorescence is related to the density of bacterial cells. The adherence of bacterial cells to the root surface also appeared in a linear manner rather than a large cluster. By observing the plant surfaces of Hysun-39, it was seen that bacteria spread on the leaf surface (Figure 2-D) whereas on the root (Figure 2-F) and stem surface (Figure 2-E) seemed to be more densely located in specific areas as the number of patches showing fluorescence are low, but fluorescence intensity is high.

CLSM also showed the special localization of bacterial cells in both varieties of various parts of *Helianthus annuus* including roots, stem and leaves as shown in Figure 3. There were no GFP-labelled bacteria found on the surfaces of uninoculated plants. However, in inoculated plants Hysun-33, GFP-labelled bacterial cells showed significant clustering in stem and inclined to be located around plant stem cell borders in a continuous manner (white arrows in Figure 3-A). The intracellular colonization of GFP-labelled bacterial cells (red arrows) was observed in plant stem cells. Under CLSM, the colonization of GFP-labelled bacteria was observed in the apoplast of leaves as well as the internal localization of bacterial cells was also seen within plant leaf cells (Figure 3-B). In roots, the colonization of bacteria was observed in the root cortex cells and intracellular localities (Figure 3-C). By observing the plant parts of Hysun-39, in Figure 3-D it could easily be seen that GFP-labelled bacteria showed localization in apoplastic spaces of leaves and penetrate within cells as well. GFP-labelled bacteria were observed in parenchymal spaces along the internal localization of cells (Figure 3-E). Significant clustering of GFP-labelled bacterial cells in roots was observed and inclined to be located around plant root cell borders and cortex cells (Figure 3-F). The picture, of the GFP-labelled phyllospheric bacterial colonization pattern, portrayed by the imaging also demonstrated the inclined behaviour of bacteria to Hysun-39 over Hysun-33. The bacteria are more densely populated in and on plant surfaces of Hysun-39, observed by the number of clusters and intensity of fluorescence (Figure 2-D-E and 3-D-E).

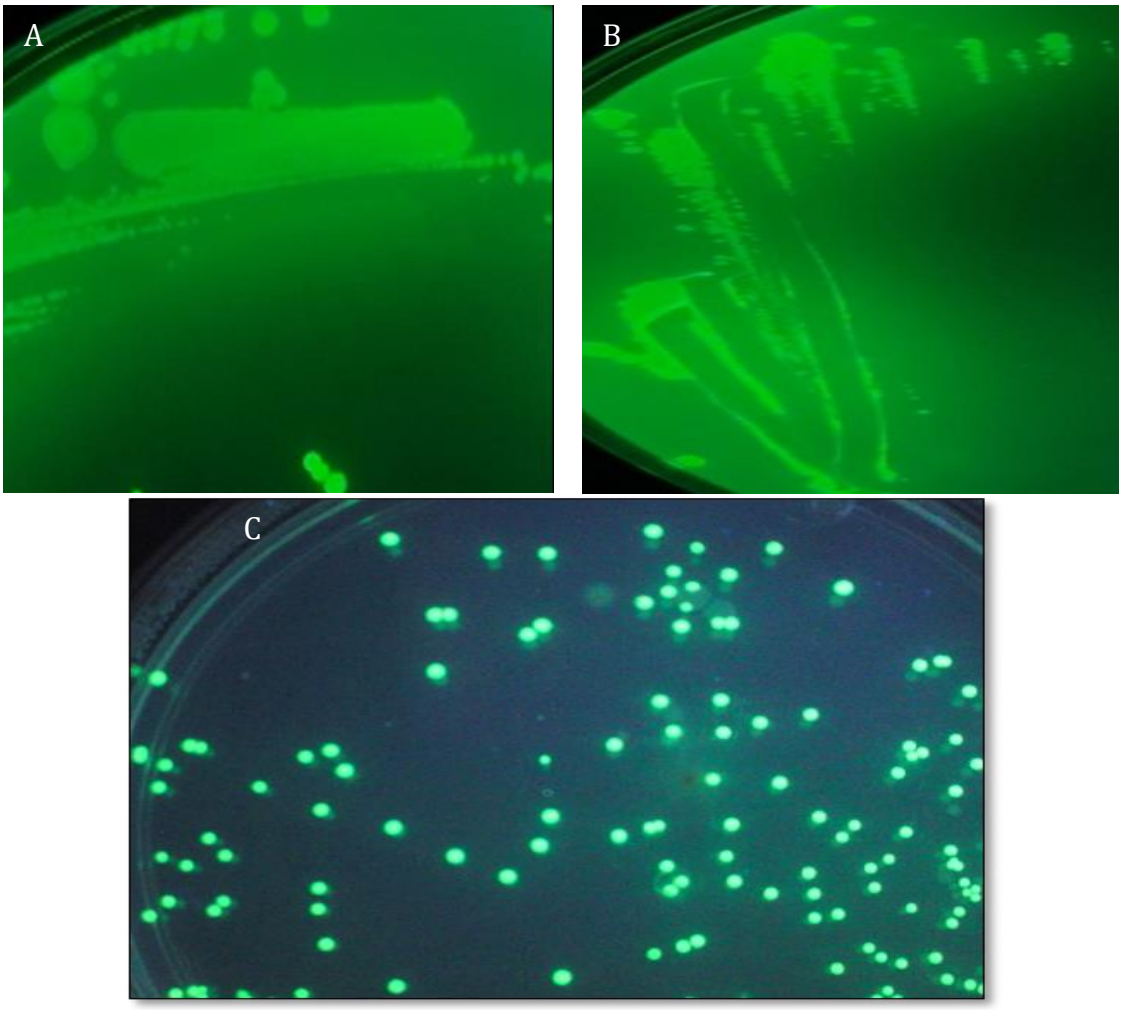
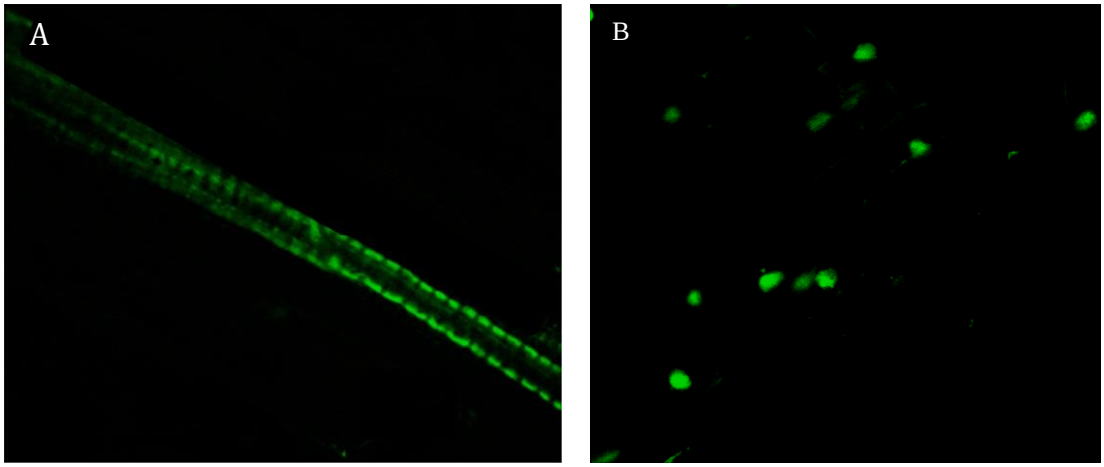


Figure 1: Detection of Green Fluorescent Protein (GFP) expression in *Pseudomonas* sp. MehA-P42 on arabinose supplemented LB media. A & B. *Pseudomonas* sp. MehA-P42 streaked plates showed green fluorescence under UV due to the expression of GFP. C. Isolated *Pseudomonas* sp. MehA-P42 colonies, confirmation of GFP expression in pure culture, under UV.



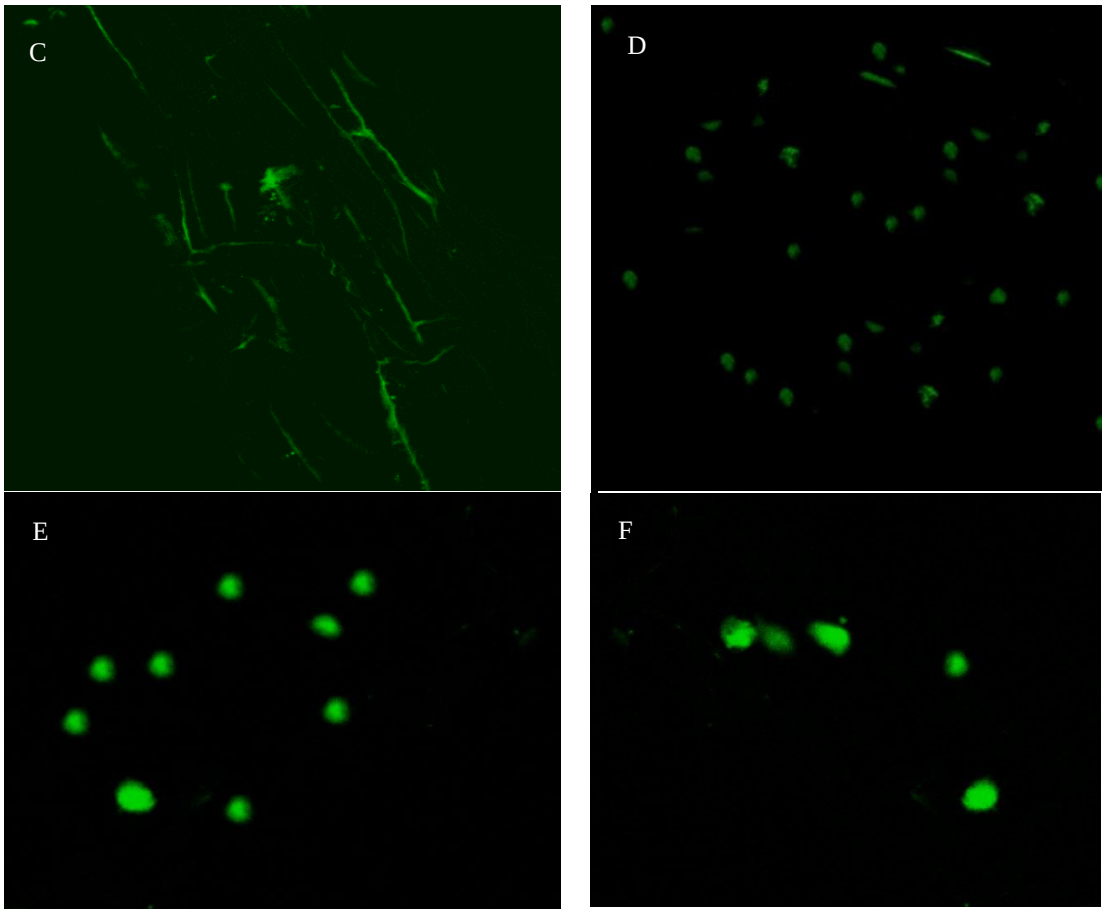
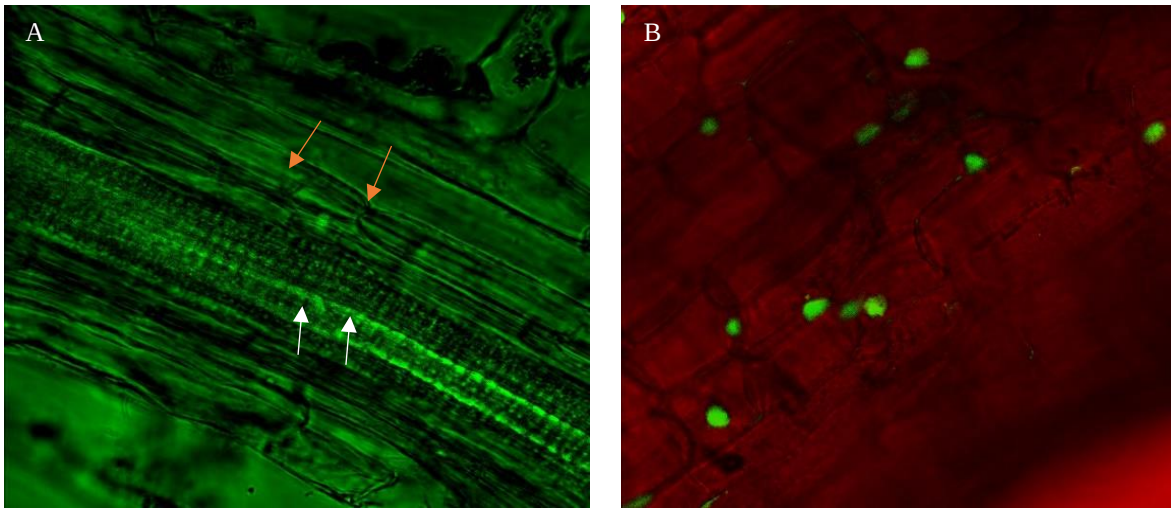


Figure 2: Fluorescent microscopy of GFP-labelled bacterial cells on *Helianthus annuus* plant parts. (A). Colonization of bacterial cells (in a row) on the stem of Hysun-33; (B). Colonization of GFP-labelled bacterial cells on the leaf surface of *Helianthus annuus* Hysun-33; (C). Colonization of GFP-labelled bacterial cells on roots of *Helianthus annuus* Hysun-33; (D). Colonization of GFP-labelled bacterial cells on the leaf surface of *Helianthus annuus* Hysun-39; (E). Colonization of GFP-labelled bacterial cells on the stem of *Helianthus annuus* Hysun-39 and (F). Colonization of GFP-labelled bacterial cells on roots of *Helianthus annuus* Hysun-39.



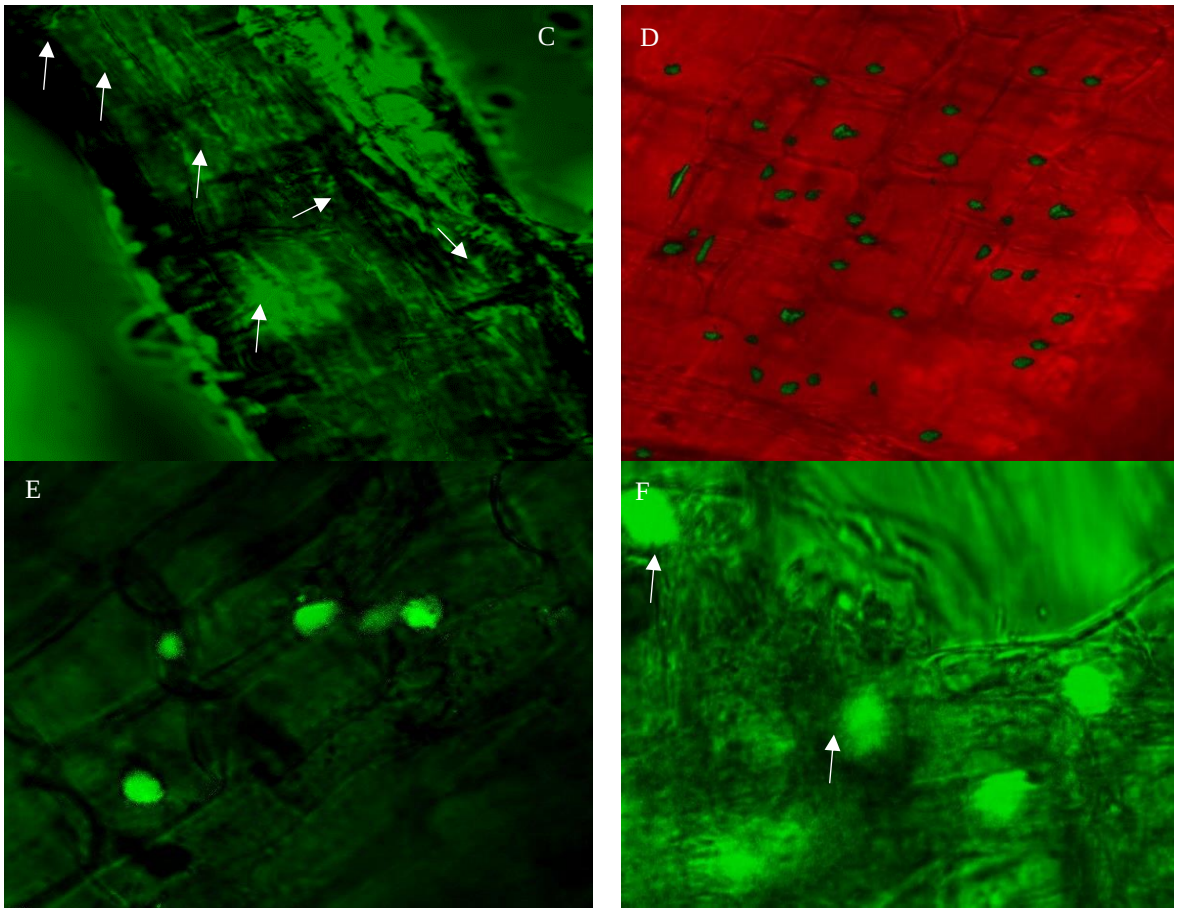


Figure 3: Localization of GFP (Green fluorescent protein) labelled bacterial cells on plant parts under confocal laser scanning microscope (CLSM). (A). Colonization of bacterial cells on the stem of *Helianthus annuus* Hysun-33; (B). Colonization of GFP-labelled bacterial cells on the leaf surface of *Helianthus annuus* Hysun-33; (C). Colonization of GFP-labelled bacterial cells on roots of *Helianthus annuus* Hysun-33; (D). Colonization of GFP-labelled bacterial cells on the leaf surface of *Helianthus annuus* Hysun-39; (E). Colonization of GFP-labelled bacterial cells on the stem of *Helianthus annuus* Hysun-39 and (F). Colonization of GFP-labelled bacterial cells on roots of *Helianthus annuus* Hysun-39.

DISCUSSION

Microbial communities' imaging is extensively used to inspect the multifaceted associations between microbes and plants (Peredo and Simmons, 2018). Green Fluorescent Protein (GFP) is a versatile biological marker for detecting transgenic expression, visualising protein localization and tracking physiological processes *in vivo* (Chen *et al.*, 2019; Werner *et al.*, 2020). Localization of GFP-labelled epiphytic phyllospheric bacteria *Pseudomonas* sp. MehA-P42, obtained from the leaf surface of *Helianthus annuus* hybrid Hysun-39 and used to inoculate the seeds of two varieties of the same plant (Hysun-33 and Hysun-39) and root, stem and leaves of both hybrids was imaged by fluorescence microscopy and CLSM (confocal laser scanning microscope). The colonization pattern of this epiphytic phyllospheric inhabitant along with the plant roots, stem and leaves surfaces of the plant was checked. Results revealed the competency of phyllospheric epiphyte towards roots and stem of both plant varieties as well. It was examined that *Pseudomonas* sp. MehA-P42 had the ability to associate and colonize the root surface and stem surface along the leaf surface. The presence of GFP-labelled bacteria on leaf surface could be due to the transmission of bacteria from seed coat to leaf during sprouting of the seed. The other possibility is more considerable that the GFP-labelled bacteria moved from roots to leaves via the stem, as the stem was also densely populated with bacterial cells. It supports the idea that phyllospheric microbiota is contributed by the rhizosphere. Bao *et al.* (2020) reported that most of the phyllospheric microbial flora is transmitted from the soil and the variation in the phyllospheric microbiota is the consequence of the open nature of leaves. The study of plant surfaces under CLSM also revealed the localization of GFP-labelled bacteria in

cortical cells of roots, parenchymal cells of stem and apoplastic regions of leaves as well as intracellular localization of cells. This showed the ability of GFP-labelled epiphyte to be endophyte by entering within plant cells. By considering this aspect, there is the possibility of the movement of GFP labelled epiphyte from roots to leaves via vascular bundles. In Figure 3-A, it could easily be seen that GFP-labelled bacteria are linearly present along with the cells, which could be due to the movement of bacteria within stem cells. Bacteria could enter within plant cells via lesions at plant body either on primary or secondary roots or any wound on plant body could act as the portal of entry or bacteria could penetrate within plant cells by utilizing plant material of leaf by secreting bacterial enzymes (cellulase or/and pectinase).

The non-uniform distribution of bacteria on the leaf surface is due to different factors, such as the lack of nutritional sources across the entire leaf surface, and the colonisation pattern depends on the combination of protective sites and the availability of nutritional resources to support efficient phylloepiphytic establishment. The combination of 3 anatomical characteristics was correlated with the most abundant phyllo epiphytic bacteria populations: the presence of active secretory cells, trichomes, and the absence of epicuticular waxes, while the opposite conditions inhibited the epiphytic establishment. (Baldotto and Olivares, 2008). In certain cases, no distinction could occur between the upper and lower leaf surfaces, despite the differential distribution of these structures in under investigation plants (Baldotto and Olivares, 2008). Here in the examination of leaves of Hysun-33 and Hysun-39, it could be seen that bacteria are not evenly distributed. The colonization of bacteria on leaves is usually held in places which are safe enough to support microbial growth like ridges, trichomes and wax. The portal of entry for internal localization of GFP-labelled bacteria in leaves could be the stomata or bacterial ability to utilize the components available in leaves to make a portal for entry.

Phyllo-epiphytic bacteria not even reached and colonized the destined aerial parts but also colonized roots and stem surface. Imaging revealed the internal localization of GFP-labelled *Pseudomonas* sp. MehA-P42 in inter and intracellular compartments of leaves, stems and roots could be considered as the attribute of extracellular enzymes released by the bacteria. Phyllospheric bacteria *Pseudomonas* sp. MehA-P42, isolated from the Hysun-39, showed the association with Hysun-33 by colonizing on and in the surfaces of root, stem and leaves. It indicated the ability of phyllo-epiphytic bacteria to quickly adapt to the environment and flourish on plants other than the native. This supports the field application of the bacterial isolates as biofertilizers, bio-stimulants, biopesticides and bioremediators.

Bacteria inhabit either roots or leaves, have the ability to provide indirect protection to plant against phytopathogens as they compete with others and make the colonization of other bacteria and phytopathogens difficult. The root associated bacteria facilitate plant regarding nutrients acquisition from soil subsequently provides additional aid to host functions (Bulgarelli *et al.*, 2013). By applying a combination of transcriptional GFP fusions and microscopy, a better understanding of bacterial colonization on the phytosphere can be achieved. Localization of phyllospheric epiphyte to roots and stem along leaves is a promising feature in promoting plant growth. Phyllosphere bacteria have been explored for their plant growth and bio-control potential and proved as good candidates in the field of agriculture due to their ability to endure the harsh environment. To introduce them in the field usually soil inoculation and seed bacterization methods are used. The ability of phyllospheric bacteria to colonize the roots and stem is a positive aspect that strengthen the idea of phyllospheric bacterial based biofertilizers. To further explore the leaf habitat, to measure microbial activity, identity, and expression of genes direct imaging can facilitate because of the cleanliness of leaves microbes can be directly seen on leaves. Microbes on leaves can be more easily observed by imaging in comparison to complex soil. Subsequently, the dynamics and distribution of microbes on leaves, and the microbial interaction with the host can be studied by the visualization techniques. Leaf-FISH can be used *in planta* 3D imaging to observe multispecies epiphytic microbial communities of leaves.

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